UNIVERSITI PUTRA MALAYSIA

EVALUATING AND TESTING OF A POTENTIAL DNA VACCINE AGAINST VIBRIO CHOLERAE

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EVALUATING AND TESTING OF A POTENTIAL DNA VACCINE AGAINST *VIBRIO CHOLERAE*

BY

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June 2003

Chairman: **Associate Professor Dr. Rozita Rosli**

Faculty: **Medicine and Health Sciences**

Although it has been more than 100 years since the first attempt to produce a cholera vaccine was made, an effective cholera vaccine has yet to be developed. In this study, the level of protection produced by a potential DNA vaccine (pVax/ctxB) was tested against the *ctxB* toxin of *Vibrio cholerae* on Balb/c mice. First, the intramuscular vaccination method was validated using pCMV plasmid that encodes HbsAg, which was detected 5 days after the injection into the tibial muscle. Next, 4 groups of mice were intramuscularly injected with either the pVax/ctxB vaccine construct or pVax1 as the negative control. The first and second groups received 2 injections spaced 3 weeks apart, while the other two groups were given 3 injections spaced 3 weeks apart. This was then followed by challenging the mice with 10^5 or 10^7 cfu/ml/mouse from clinical isolates of *V. cholerae* after 3 weeks of the last injection. Antibody levels for both IgG and serum IgA were monitored using ELISA, and showed high production of IgG after the first booster injection with no significant change of IgA levels. However, after the second

booster injection, the antibody levels for both IgG and IgA declined. This was accompanied by the death of 2 mice in the first vaccinated group, and all the mice in the control group after the bacterial challenge with 10^7 cfu/ml/mouse. In the second group, none of the mice survived in both vaccinated and control groups. The bacterial challenge using 10^5 cfu/ml/mouse failed to induce the death in all the groups.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENILAIAN DAN PENGUJIAN POTENSI VAKSIN DNA TERHADAP *VIBRIO CHOLERAE*

Oleh

LAMA ABDEL QADER MOH'D HAMADNEH

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Pengerusi: **Profesor Madya Dr. Rozita Rosli**

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Walaupun setelah lebih dari pada 100 tahun semenjak percubaan pertama menghasilkan vaksin taun dilakukan, namun sehingga kini penghasilan vaksin yang benar-benar efektif masih belum menampakkan hasil. Bagi kajian ini pula, tahap ketahanan yang dihasilkan oleh calon vaksin DNA (pVax/ctxB) telah diuji menentang toksin ctxB dari *Vibrio cholerae* dengan tikus Balb/c sebagai hos. Buat permulaan, teknik vaksinasi intramuscular telah di sahkan dengan menggunakan plasmid pCMV yang mengkodkan HbsAg yang mana telah dikesan setelah disuntik ke otot tibial 5 hari kemudian. Empat kumpulan tikus telah disuntik menggunakan teknik di atas samada dengan vaksin pVax/ctxB atau pVax sebagai kawalan negatif. Kumpulan pertama dan kedua menerima 2 suntikan setiap 3 minggu manakala 2 kumpulan lagi 3 suntikan bagi setiap 3 minggu. Langkah berikutnya, setiap kumpulan ditentang dengan 10^5 atau 10^7 cfu yang telah dipencilkan secara klinikal dari *V.cholera*. Paras antibodi bagi kedua-dua IgG dan serum IgA dipantau menggunakan ELISA. Keputusan ELISA menunjukkan paras

antibodi IgG tinggi sementara tiada perubahan ketara penghasilan paras IgA setelah suntikan booster pertama. Setelah suntikan booster kedua, paras antibodi IgG dan IgA didapati menurun. Keadaan ini diikuti dengan kematian 2 ekor tikus dari kumpulan pertama yang divaksin dan kematian kesemua tikus kumpulan kawalan setelah ditentang dengan 10^7 cfu *V. cholerae* yang virulen. Bagi kumpulan kedua, kesemua tikus (vaksin dan kawalan) mati. Tentangan bakteria 10^5 gagal untuk menyebabkan kematian bagi kesemua kumpulan.

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I certify that an Examination Committee met on 20th June 2003 to conduct the final examination of Lama Abdel Qader Moh'd Hamadneh on her Master of Science thesis entitled "Evaluating and Testing of a Potential DNA Vaccine against *Vibrio cholerae*" in accordance Universiti Pertanian Malaysia (Higher Degree) act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and was accepted as fulfillment of the requirements of the degree of Master of Science. The members of the Supervisory Committee are as follows:

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I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

LAMA ABDEL QADER MOH'D HAMADNEH

Date: 26/7/03

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LIST OF ABBREVIATIONS

Ace	Accessory cholera enterotoxin
ACF	Accessory colonization factor
AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cells
BSA	Bovine serum albumin
cAMP	Cyclic adenosine 51-monophosphate
Cep	Core encoded pilin
CpG	Cytosine-phosphate-guanosine
CPS	Capsular polysaccharide
CTL	Cytolytic T lymphocytes
Ctx	Cholera enterotoxins
DNA	Deoxy ribonucleic acid
EDTA	Ethylene Diamine tetra acetic acid
ELISA	Enzyme linked immunosorbant assay
GM-CSF	Granulocyte-monocyte colony stimulating factor
HA	Haemagglutinin
HBsAg	Hepatitis B surface antigen
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
H_2SO_4	Sulfuric acid
ID	Intradermal
Ig	Immunoglobulin

IL	Interleukin
IM	Intamuscular
INF	Interferon
KCl	Potassium chloride
LB	Lauria-Bertani
LPS	Lipopolysaccharides
LT	Heat labile enterotoxins
MFRHA	Mannose-fucose-resistant hemagglutinin
MHC	Major histocompatability complex
mRNA	Messenger ribonucleic acid
MSHA	Mannose-sensitive-hemagglutinin
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicabonate
NK	Natural killer
NP	Nucleoprotein
OMP	Outer membrane proteins
ORS	Oral rehydration salts
PBS	Phosphate buffer saline
PLG	Poly (lactide-coglycolide)
TCBS	Thiosulfate-citrate-bile salts-sucrose.
TCP	Toxin-coregulated pili
Th	T helper
TMB	Tetramethylbenzidine

WHO	World Health Organization
Zot	Zonula occludens toxin

CHAPTER 1

INTRODUCTION

Around 200 years ago, immunization against infectious diseases was started when Edward Jenner published his method of preventing smallpox in 1798 (1). Although it took nearly 100 years before the appearance of the next vaccine in 1880, vaccine development was rapid, where in 1884, the first attempt to produce a parenteral cholera vaccine of broth cultures of *Vibrio cholerae* was reported by Ferran (2). However, after more than 100 years of this first vaccination attempt, no effective cholera vaccine is yet available to date.

The cholera disease can spread easily by water and food contamination, causing severe diarrhea and death among people living with poor sanitary facilities. Wars and political unrest, climate changes and natural catastrophes, increase the number of people under the threat of cholera epidemics. Furthermore, these conditions are still prevailing in many parts of the world. On the other hand, the increasing number of geographical areas becoming endemic for cholera reflects a failure of implementation of control measures. Water and electrolytes replacement therapy is not fast enough during times of outbreak, and misguided use of antibiotics has led to the emergence of multi-resistant strains, some of which were found to be highly virulent (3).

As of September 2002, 106,547 cases with 3,155 deaths were officially reported to the World Health Organization (WHO) (4). In the year 2001, Malaysia reported 557 cases with 11 deaths. From a total of 184,311 cases 2,728 deaths were reported from the whole world (5).

The need for an effective cholera vaccine is urgent to produce high level of protection in people living in high-risk areas. A parenteral vaccine based on inactivated *V. cholerae* O1 has been available for more than 40 years. Nevertheless, this vaccine is not recommended any more by WHO, since its protective efficacy is modest, of short duration and it does not prevent the transmission of *V. cholerae* (3).

At present, new cholera vaccines are under development, and 2 oral vaccines are already available internationally, WC/rBS and CVD103-HgR, which conferred good protection in adults. But thus far, all the vaccines being used give only 50% protection after 6 months of immunization together with no sustained protection in children under 2 years (3).

In early 1990's, the ability of inserted genes into plasmid vectors was studied to prompt an immune response; surprisingly the immunity elicited was strong enough to protect against infectious diseases. The immune response produced by these vaccines, which are termed as DNA vaccines (unlike that produced by conventional vaccines) can stimulate both humoral and cellular immune responses. The immune response produced was found to be long lasting and it might overcome the deficits of

the conventional vaccines used nowadays. In addition, new protection against diseases like AIDS, malaria, and hepatitis C can be provided.

The field of DNA vaccines is developing rapidly. Today, many clinical trials are conducted to test the efficacy of DNA vaccines against HIV, hepatitis B and some tumors such as B-cell lymphomas. Despite that, any new vaccine should be first tested on small animals to detect the immunogenicity of the antigen expressed, followed by testing its protective properties against the challenge. In addition, before proceeding to human clinical trials, the vaccine should be tested on higher animals to adjust the dose and the boosting pattern.

In an attempt to develop a cholera DNA vaccine, the gene encoding for the B subunit of the enterotoxin AB was cloned in pVax1 plasmid vector in a previous study (6), followed by successful *in vitro* expression of the antigen using COS-7 cell line.

Hence, this study focuses on the next step, in which the ability of this potential vaccine to be expressed and elicit an immune response *in vivo* is tested, or in other words to test the antigen`s immunogenicity. *In vivo* tests based on measuring the level of antibody production and the protection level against the challenge with clinical isolates of *Vibrio cholera* after intramuscular immunization of this potential cholera vaccine were conducted using female BALB/c mice.

Objectives

(1) To validate the intramuscular injection technique using pCMV-S plasmid that carries HBsAg in Balb/c mice.

(2) To test the cholera DNA vaccine (pVax/ctxB) intramuscularly by measuring the levels of both IgG and IgA produced after each vaccination and compare it to a negative control plasmid (pVax1) without any insert.

(3) To determine the efficacy of pVax/ctxB in conferring protection or immunity against the challenge with either 10^5 or 10^7 cfu/ml/mouse of clinical isolates of *Vibrio cholerae*.

CHAPTER II

LITERATURE REVIEW

Cholera

History

As early as the time of Hippocrates and Galen, cholera has sporadically affected humans all over the world where some records from this time described cholera-like symptoms. However, modern knowledge about cholera started in the beginning of 19th century, where the English physician John Snow in 1849 indicated the importance of water as the carrier of the disease. In 1883, Robert Koch succeeded to isolate *Vibrio cholerae* from the intestinal discharges of cholera patients.

Cholera has smoldered in an endemic fashion on the Indian subcontinent for centuries, from where the first long-distance spread of the disease to Europe and the Americas began in 1817 and by the early 20th century, six waves of cholera had spread all over the world. The 7th cholera pandemic caused by the *El Tor* biotype was started in 1961 from Indonesia and spread rapidly to Asia, Europe, Africa and finally in 1991 to Latin America, which was free of cholera for more than a century. The 7th pandemic has not receded; on the contrary, cholera has now become endemic in many parts of the world (7). Also, since 1992, *V. cholerae* O139, a new and more virulent serogroup variant of *El Tor* biotype has spread to many parts of Asia (3).

Disease

Cholera is an acute gastrointestinal disease where the production of diarrhea is the main symptom. The disease can range from mild in most cases with less than 1% mortality to a severe life threatening infection termed as cholera gravis with more than 50% mortality (3, 8). The symptoms might appear as sudden with profuse watery diarrhea or there can be some premonitory symptoms like anorexia, abdominal discomfort and simple diarrhea.

Initially the stool passed is brown, but soon it assumes a pale gray color with an inoffensive, slightly fishy odor. Mucus in the stool imparts its characteristic rice water appearance. Resultant water and electrolyte loss leads to thirst, muscle cramps, weakness, and sunken eyes. In cholera gravis, the rate of water loss may reach 1 L/hr leading to tachycardia, hypotention and vascular collapse due to dehydration (9, 10). If untreated severe metabolic acidosis with potassium depletion, anuria, circulatatory collapse and cyanosis can occur, leading to death.

Major alterations in mental status are uncommon in adults; the patient usually remains well oriented but apathetic, while hypoglycemia, coma and convulsions might occur in children. After recovery, very small minority of patients (< 1%) continue to carry the pathogen in the gallbladder and excrete it with stools; however, most patients are free after about 2 weeks (10).

Mode of Transmission

Cholera infection usually begins with the ingestion of contaminated food or water. Food can buffer the acidity of the stomach thus decreasing the infecting dose of the bacteria as well as providing an ideal culture medium (11). Furthermore, sea food can acquire the bacteria from the environmental sources and causes outbreaks or sporadic cases specially if it is uncooked or partially cooked (12).

Incubation Period and Infectious Dose

The incubation period can range from a few hours to 5 days, usually 2-3 days and it is dependent in part on the inoculum's size (13), where 10^3 of bacteria in the intestine is enough to start the infection. It is estimated that around 10^{11} bacteria are required as an infective dose in normal gastric acidity individuals (14).

Age and Susceptibility

In endemic areas, the disease is concentrated more in children aged 2 to 9 and in women in their child bearing years (15-35 years) where there is a decrease in immunity and the exposure to the environment is higher. On the other hand, infants under 1 year of age are protected because of breast feeding (15). Individuals with blood group O are at higher risk for cholera gravis due to El Tor biotype and O139 V*ibrio* although the mechanism responsible for this difference is not known (16); also, individuals with gastric achlorhydria have a higher risk for getting the disease.